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13. ABSTRACT ***(Maximum 200 Words)***

The focus of our USAMRMC sponsored investigations is the study of mammary myoepithelial cell function and the role of these cells in regulating glandular development and differentiation during pregnancy. We hypothesize that myoepithelial cells, through elaboration of the ECM and via integrin-based signaling cascades regulate mammary development and differentiation. We have produced a transgenic animal model in which a K5 promoter directs expression of the E2F-1 transgene to mammary myoepithelial cells. Our characterization of whole mounts revealed that glands from virgin transgenics had profoundly reduced ductal arborization and branching compared to those from wild types. Proliferation was decreased and apoptosis increased in the transgenic glands, compared to wild types. Alveolar development was severely curtailed in glands harvested from pregnant transgenics, and numbers of myoepithelial cells in the mammaries were reduced. Taken together, these data suggest that E2F-1 over expression induces apoptosis of myoepithelial cells, and this loss of myoepithelial control affects ductal development and inhibits the acquisition of the differentiated phenotype of pregnancy. Since the structural and functional differentiation achieved during gestation is protective against breast cancer, the myoepithelial cells can be considered mediators of the susceptibility of the glands to tumorigenesis and novel targets for preventive and therapeutic strategies.

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Table of Contents

Introduction

The focus of our USAMRMC sponsored investigations is the study of mammary myoepithelial cell function and the role of these cells in regulating both glandular development and differentiation during pregnancy. Myoepithelial cells are found in the adult mammary gland surrounding the ducts and acini and on cross section are seen as a basal layer of spindle shaped cells underlying the luminal epithelium. The myoepithelial cells lay down basement membrane (BM), express cytokeratins 5 and 14, but not 8 and 18, and are responsible for maintaining the integrity of the ductal structure.

Studies indicate that the myoepithelium and its product basement membrane regulate differentiation of the mammary gland during pregnancy. Specific components of the extracellular matrix regulate integrin-based signal transduction cascades resulting in gene activation, leading to changes in expression of milk proteins (1). *In vitro* studies demonstrate that β-casein production requires the presence of a laminin-rich matrix.

Numerous reports also indicate that pregnancy and accompanying glandular differentiation are protective against cancer in humans and rodents (2). In rodent studies, a single pregnancy affords significant protection against subsequent treatment with chemical carcinogens, reducing tumor incidence by 30-50% (3). Epidemiologic studies suggest that early pregnancies also affords mammary protection in humans, and it has been speculated that multiparity seen in rural cultures may account, in part, for reduced breast cancer incidence (4).

Hence, the hypothetical basis for our work is that the myoepithelial cells that produce the ECM are a primary regulator of tissue differentiation and are, therefore, mediators of the susceptibility to tumorigenesis. We have developed a transgenic animal model to study myoepithelial cell function, in which over expression of E2F-1 is controlled by the K5 promoter and is limited to the myoepithelial cells.

Body

Our preliminary data showed that the K5 E2F-1 transgenic animals were unable to nurse and that glands from pregnant transgenics were hypoplastic and lacked alveolar development. We proposed to further characterize glands from wild type and transgenic animals by analysis of whole mounts and immunohistochemical localization of K5. Also to be completed within the first 15 months of the grant, were a comparison of proliferation levels, as measured by immunolocalization of BrdU-incorporating cells, and apoptosis, as determined by TUNEL assays.

A comparison of the whole mounts of glands harvested from age-matched virgin animals revealed that the glands from K5 E2F-1 transgenics had severely diminished ductal development. As shown in figure 1, the ductal arborization in transgenics is greatly reduced, the ducts are coarser and thickened and there is a profound lack of cross branching.

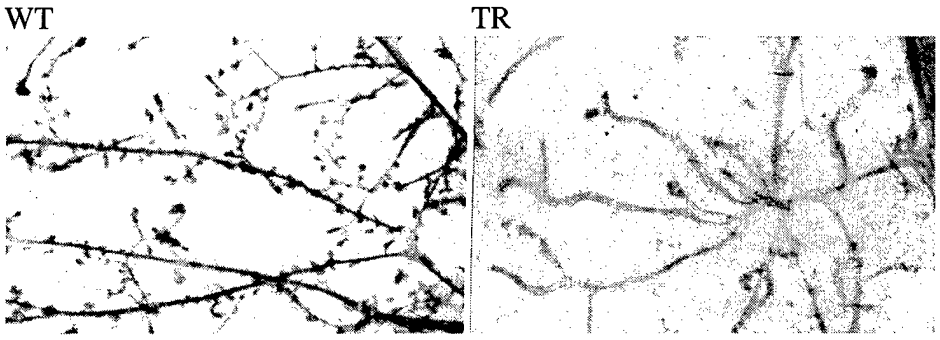

Figure 1. Whole mounts showing ductal structure of sexually mature virgin animals. Glands from wild type (WT) on the left or transgenic (TR) on the right are pictured. Virgins were staged by vaginal cytology and harvested in the estrus phase of the cycle. Mammary glands were isolated, pressed between two pieces of glass, fixed in buffered formalin, defatted and stained with toluidene blue.

The ductal dilation seen in the transgenics can also be appreciated in H&E stained cross sections shown in figure 2.

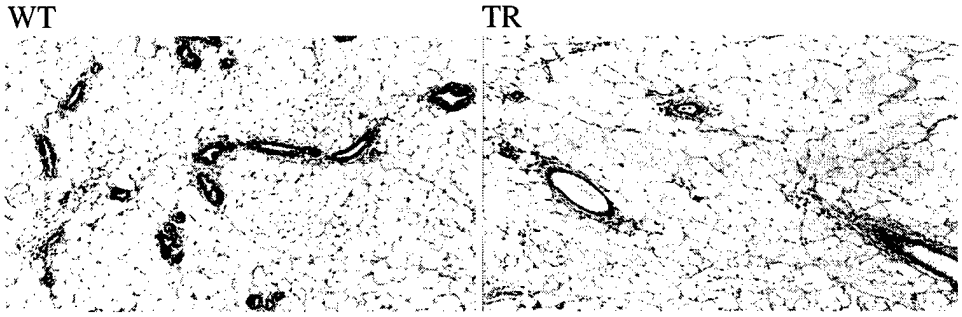

Figure 2. H & E stained sections of mammary glands harvested from sexually mature, virgin animals, wild type (WT) and transgenic (TR).

Histological and morphological analysis of glands harvested from pregnant animals on the day of parturition (lactation day 1) also reveal substantial differences between wild types and transgenics. As shown in figure 3, H&E stained sections indicate that E2F-1 overexpression in mammary myoepithelial cells inhibited full glandular differentiation during pregnancy. Glands from transgenic animals were hypoplastic and had fewer alveoli and increased amounts of interductal fat as compared to wild types. This observation is important in light of the fact that transgenic females were fertile and able to support a normal gestation and parturition, indicating a normal hormonal milieu. These results indicate that the changes in mammary development were not due to endocrine deficiencies but instead were due to a direct effect of K5 directed overexpression in myoepithelial cells.

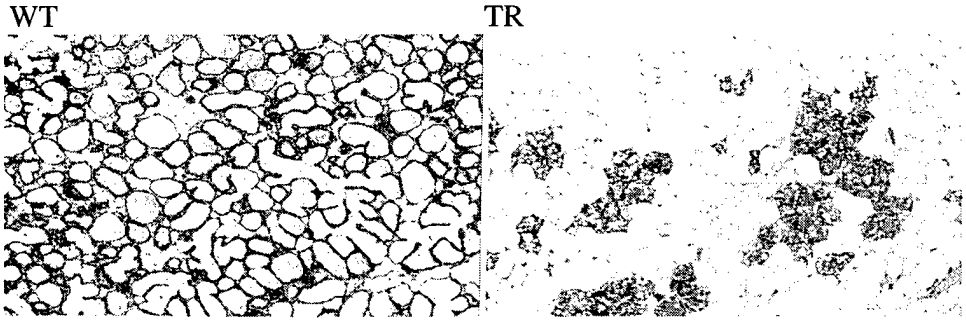

Figure 3. H & E stained sections of mammary glands harvested from mice on the day of parturition (lactation day 1). Study mice were bred and monitored during the 3 week gestation period. Mothers were sacrificed on the day the pups were born, usually within 3 hours.

Analyses of BrdU incorporation revealed that mammary glands harvested from transgenic animals had reduced levels of proliferation compared to age- and parity-matched wild types. As seen in figures 4 and 5, the percentage of cells that incorporated BrdU was higher in mammary glands harvested from wild type animals compared to the transgenics, in both virgins and pregnants. Although this difference is visually evident in histological sections of virgin animals (figure 4), the data did not reach statistical significance due to animal-to-animal variation (figure 5). It is anticipated that this difference will reach statistical significance when additional tissues are analyzed, as a trend towards reduced proliferation in glands from virgin transgenics is evident.

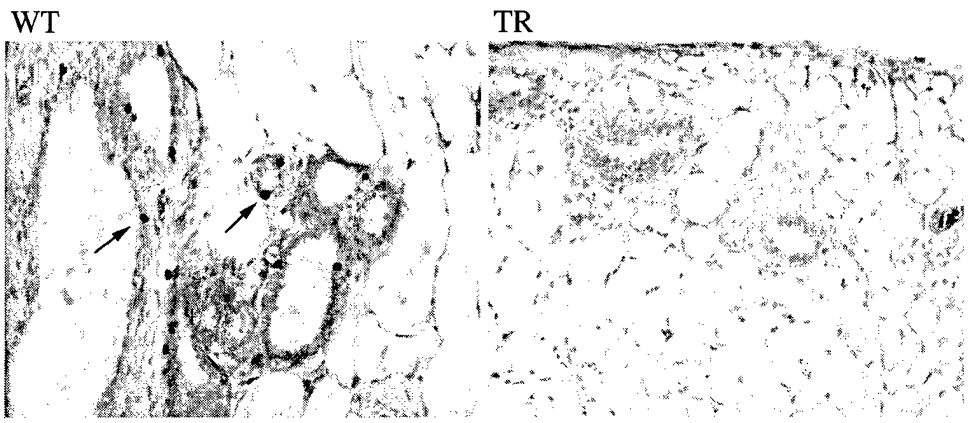

Figure 4. BrdU incorporation in glands from virgin wild type (WT) and transgenic (TR) mice. Arrows show BrdU incorporating, positive-staining cells.

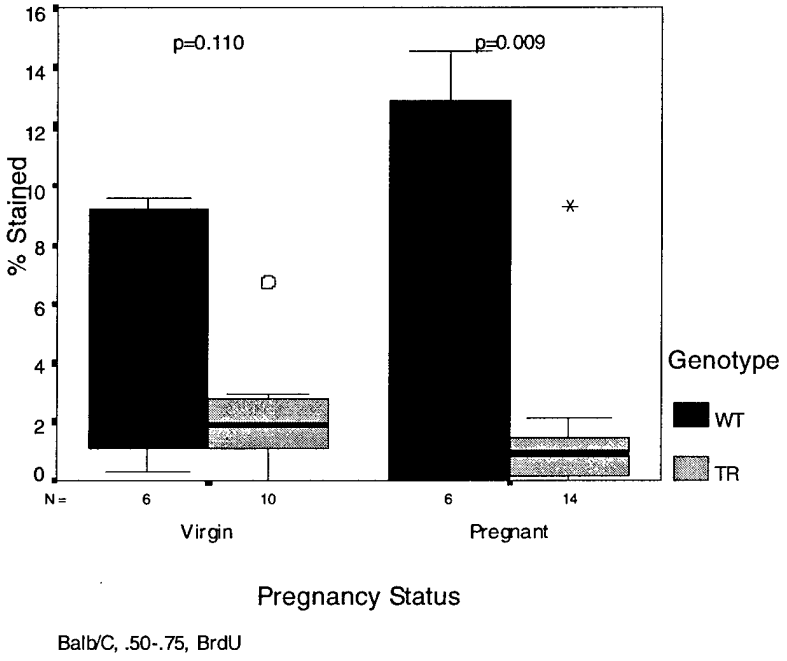

Figure 5. Quantitation of BrdU incorporating cells in glands harvested from wild type (WT) and transgenic (TR) animals. Statistical significance was determined by application of Student's t test.

As proposed, apoptosis was also compared in the glands taken from wild type and transgenic animals using TUNEL analysis. Although the number of TUNEL-positive cells were relatively low in all glands, samples from virgin transgenic animals had higher levels of apoptosis compared to wild types (figures 6&7). This increased amount of apoptosis in transgenic glands was especially marked in pregnant animals (figure 7), suggesting that the hormonal, morphological or structural changes that take place during gestation may play a role in stimulating programmed cell death. This observation is consistent with the original hypothesis that the myoepithelium plays a seminal role in the acquisition of fully differentiated structure and function during pregnancy.

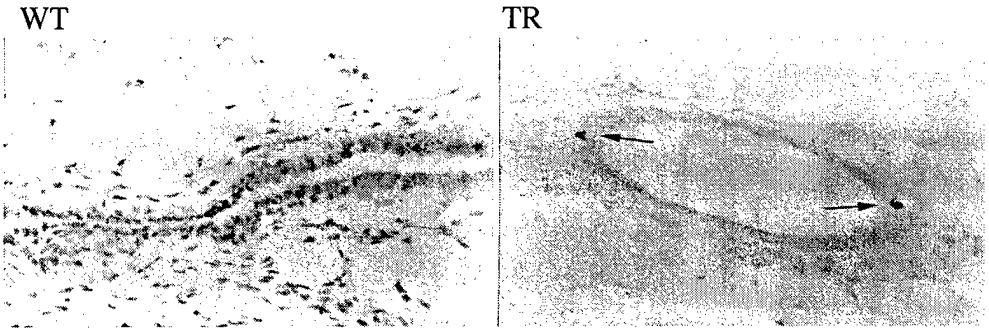

Figure 6. Apoptosis in glands from virgin mice. Arrows indicate TUNEL-positive cells in transgenic glands.

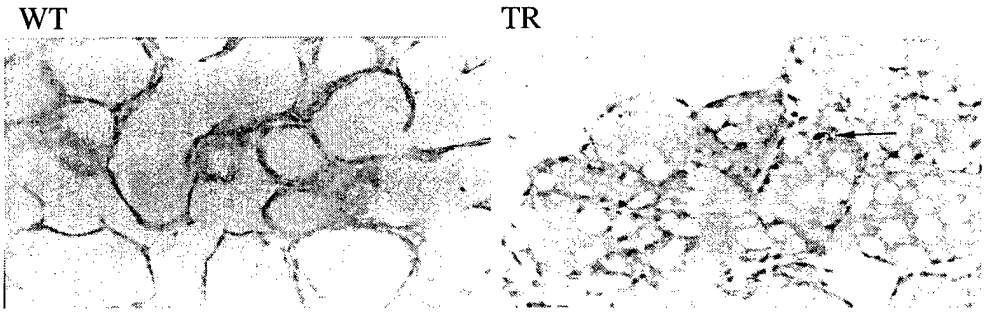

Figure 7. Apoptosis in glands from pregnant mice. Arrow indicates TUNEL-positive (apoptotic) cell in glands from a transgenic mouse.

Quantitation of apoptosis in glands from study animals is shown in figure 8. Although the percentage of TUNEL-positive cells was higher in both virgin and pregnant transgenics compared to wild types, only the data from pregnant animals reached statistical significance at the time of this report. We feel that this is, in part, due to the low level of apoptosis found in glands from virgin animals. Increased numbers of study animals are currently being produced and it is anticipated that differences will become statistically significant if present trends continue.

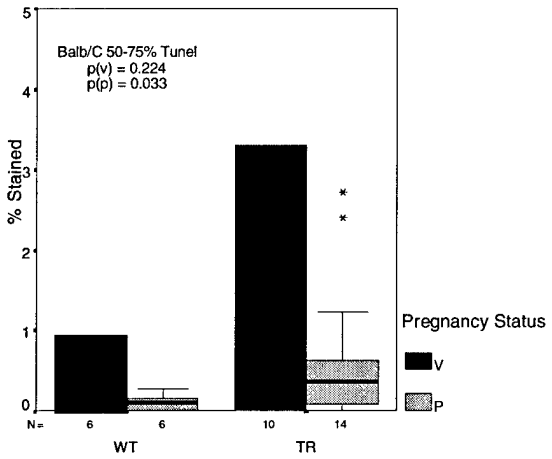

Figure 8. Quantitation of apoptosis in wild type (WT) and transgenic (TR) animals. Glands from virgins (V) or pregnant (P) animals were harvested and fixed. Apoptotic cells were detected using the apotag detection kit.

Immunolocalization of K5 in the glands from virgin and pregnant animals, shown in figures 9 & 10, provides insight into the impact of transgene overexpression on the myoepithelial cells themselves. In the mammary gland, the myoepithelial cells exclusively express keratin 5. Thus, immunostaining in

these sections indicates the location and integrity of the myoepithelial layer. In the virgin glands, the smooth myoepithelial layer appears partially disrupted, and staining of cell bodies is more discrete than seen in the wild type glands. In the transgenic pregnant animals the number of positively stained cells is greatly reduced, suggesting a loss of myoepithelial cells over the course of gestation.

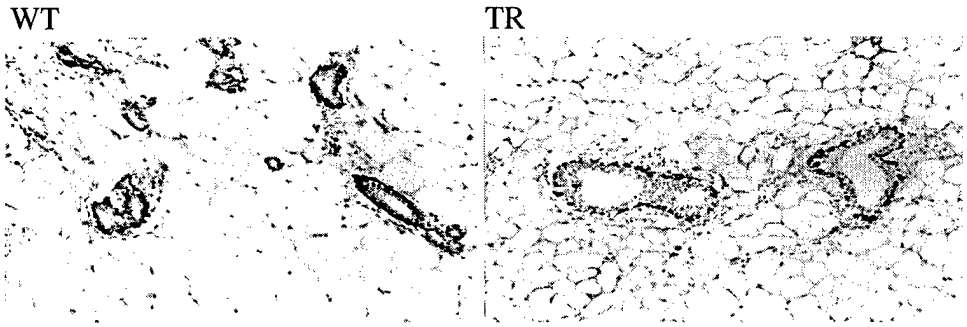

Figure 9. K5 staining in virgin glands. Myoepithelial cell bodies surrounding the ducts are positively stained.

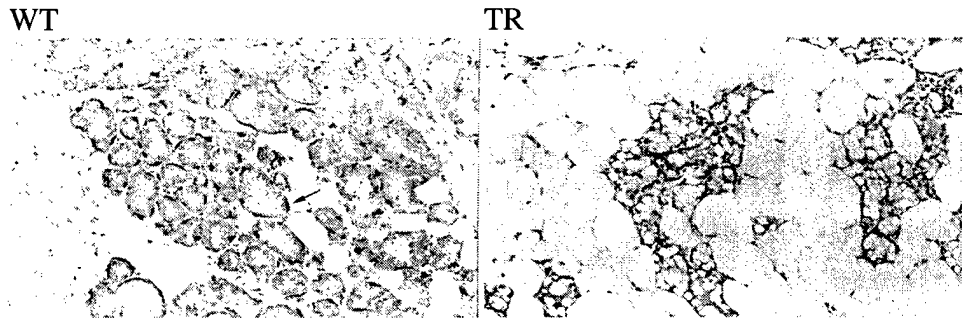

Figure 10. K5 staining in pregnant glands.

At this time, our interpretation of this data is that overexpression of E2F-1 induces apoptosis of the myoepithelial cells which are not readily replenished from a pool of progenitors. The ability of overexpression of E2F-1 to stimulate apoptosis has been reported and discussed by others (reviewed in (5). The apoptotic loss of myoepithelial cells impacts the structural development and function of the gland at multiple levels, leading to reduced proliferation, lack of alveolar expansion and failure to develop a fully differentiated pregnant state. Although we do not yet know the mechanism by which this occurs, the data support the original hypothesis proposed in the application, that myoepithelial cells play a pivotal role in regulating mammary development and differentiation in both virgin and pregnant animals.

Key Research Accomplishments

During the previous budget period we have successfully :

- **Developed appropriate breeding strategies and expanded the animal colony to provide necessary animals for analysis. (Task 1)**
- **Produced animals and evaluated glands from 2 of the 4 timepoints (0 and 3 weeks of gestation completed). (Task 2)**
- **Developed appropriate methods of tissue harvest and produced whole mounts, embedded sections, slide blanks and H&E sections of sufficient quality for analysis. (Task 3)**
- **Performed immunostaining of sections for K5, BrdU and TUNEL. (TASK 4)**

In addition, our data demonstrate the following:

- Histological and morphological analyses of mammary glands harvested from age- and parity-matched wild type and transgenic animals with targeted transgene overexpression and disrupted myoepithelial cell function are characterized by:
  - stimulation of increased, abnormal levels of cell death,
  - suppression of proliferation, and
  - net loss of myoepithelial cells.

- Disruption of myoepithelial function leads to deficient development of mammary glands in transgenic animals, demonstrated by:
  - lack of complete ductal arborization in virgins, and
  - hypoplasia and lack of alveolar development in pregnants.

Reportable Outcomes

To date, the work supported by this award has yielded two abstracts leading to presentations at national meetings (American Association for Cancer Research and The Endocrine Society). Copies of the abstracts are included in the appendix. We are currently preparing a manuscript describing our scientific results that will be submitted to a top scientific journal within the next budget period.

Conclusions

Our data support the original hypothesis that states that myoepithelial cells are important regulators of mammary development and differentiation. This is demonstrated by the fact that loss of myoepithelial function due to transgene overexpression resulted in reduced ductal complexity in virgin animals and lack of fully differentiated structure and function in pregnant animals.

Importantly, this award has supported the characterization of a relevant, new animal model that allows evaluation of the role of myoepithelial cells in mammary gland biology. This model is unique; there are no other appropriate models that allow evaluation of myoepithelial cell function and the impact on glandular development *in vivo*.

As discussed in the original application, the structural and functional differentiation achieved during pregnancy is protective against breast cancer. Since the myoepithelium demonstrably regulates acquisition of glandular differentiation, these specialized cells can be appreciated as mediators of the susceptibility of the glands to carcinogenesis. We believe that these cells are, therefore, appropriate targets for novel preventative and therapeutic strategies to fight this deadly disease.

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Appendices

1. Abstract of presentation at 90th Annual meeting of the American Association for Cancer Research. Overexpression of E2F-1 and *c-myc* in mammary myoepithelial cells inhibits glandular differentiation and lactation. Fuchs-Young, R., Gamage, S., Ramirez, I., Gimenez-Conti, I., Conti, C., Johnson, D.

2. Abstract of presentation at 82nd Annual Meeting of The Endocrine Society. Transgenic mouse models indicate that myoepithelial cells regulate mammary development and differentiation. Fuchs-Young, R., Gamage, S., Johnston, D., Gimenez-Conti, I., Johnson, D., Conti, C.

Abstract of presentation at 90th Annual meeting of the American Association for Cancer Research, April 10-14, 1999, Philadelphia, PA.

Overexpression of E2F-1 and c-*myc* in mammary myoepithelial cells inhibits glandular differentiation and lactation. Fuchs-Young, R., Gamage, S., Ramirez, I., Gimenez-Conti, I., Conti, C., Johnson, D. UT/MD Anderson Cancer Center, Dept. of Carcinogenesis, Smithville, TX 78957.

Studies show that pregnancy at an early age is protective against breast cancer in humans and animals. The precise mechanisms underlying this protection are unclear, but increased structural and functional differentiation of the glands is involved. To study the role of myoepithelium in regulating differentiation and cancer susceptibility, we developed transgenic mice in which E2F-1 or c-*myc* overexpression is controlled by the K5 promoter and is directed to the myoepithelial cells of the mammary gland. Transgenic females from both lines were fertile but unable to nurse pups. Glands from post-pubertal, virgin E2F-1 transgenics had profoundly reduced ductal branching and alveolar bud formation compared to wild type littermates. Mammary glands from E2F-1 transgenics had significantly increased levels of apoptosis but reduced proliferation. Glands from pregnant E2F-1 transgenics were hypoplastic, had reduced alveolar development, increased interductal fat and lacked myoepithelial cells. These data suggest that overexpression of E2F-1 stimulated apoptosis and inhibited proliferation leading to loss of the myoepithelium. In contrast, mammaries from c-*myc* transgenics had decreased levels of both apoptosis and proliferation. Virgin glands had normal amounts of ductal branching but no buds. Myoepithelial cells were present in glands from pregnant *c-myc* transgenics but alveolar morphology was disrupted and glandular epithelium attenuated. These results indicate that the myoepithelium plays a pivotal role in directing structural and functional differentiation of the mammary before and during pregnancy, and may, therefore, be an important mediator of susceptibility to carcinogenesis.

Abstract of presentation at 82nd Annual Meeting of The Endocrine Society, June 21-24, 2000, Toronto, Canada.

TRANSGENIC MOUSE MODELS INDICATE THAT MYOEPITHELIAL CELLS REGULATE MAMMARY DEVELOPMENT AND DIFFERENTIATION. R. Fuchs-Young, S. Gamage, D. Johnston, I. Gimenez-Conti, D. Johnson, C. Conti. UT MD Anderson Cancer Center, Science Park Research Division, Smithville, TX 78957

Studies indicate that pregnancy is protective against mammary cancer in rodents and humans, and that this effect is associated with structural and functional differentiation. To study the role of myoepithelium in achieving the protective effects of pregnancy, we have developed transgenic mice in which overexpression of E2F-1 or c-*myc* in the mammary gland occurs exclusively in myoepithelial cells. Although female transgenics are fertile, overexpression of E2F-1 or c-*myc* disrupted myoepithelial function, inhibited mammary differentiation and rendered mothers unable to nurse their pups. Glands from pregnant TR females were hypoplastic and underdeveloped compared to wild type (WT) littermates. In K5 E2F-1 transgenics, virgin glands had severely diminished ductal development and branching. Glands from pregnant E2F-1 TRs had greatly reduced alveolar development and increased interductal fat compared to WTs. TUNEL analyses indicated that E2F-1 overexpression induced apoptosis of myoepithelial cells, which were virtually absent in glands harvested shortly after parturition, suggesting that these necessary cells were not readily replenished. In c-myc transgenics, glands from virgin animals were mildly affected, but glands from pregnant TR mice were highly disorganized with disrupted alveolar structure. In WT animals, an increase in proliferation and a decrease in apoptosis accompany development of the lactational phenotype, but the opposite was seen in tissues harvested from c-*myc* transgenics. In pregnant c-*myc* TR animals there was no significant increase in mammary BrdU incorporation but TUNEL-positive cells were significantly increased compared to virgins. Analyses of 4 congenic strains showed significant differences in the magnitude of transgene-induced alterations, although trends of decreased proliferation and increased apoptosis were similar. These results indicate that myoepithelial cells are important regulators of the development of the fully differentiated mammary phenotype of pregnancy, and may do so by mediating the balance of proliferation and apoptosis in glandular tissue. This effect is likely regulated by myoepithelial cells through control of extracellular matrix production and content. Since myoepithelial cells regulate the development of the protective lactational phenotype, this suggests that they are important mediators of the susceptibility of the gland to tumorigenesis and potential targets of therapeutic or protective strategies.